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TITLE: A Novel Approach for Effectively Treating SCI Pain, Improving Opioid Efficacy, and Preventing Opioid-Induced Constipation: Key Role of Toll-Like Receptor 4 (TLR4)

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14. ABSTRACT
Spinal cord injury (SCI) is a disabling and costly condition affecting wounded military personnel (1). SCI is also one of the leading causes of central neuropathic pain, and military personnel that sustain SCI in the field by improvised explosive devices (IEDs), grenades, gunshot wounds, etc. are at an even greater risk of developing chronic pain as well as emotional symptoms due to the polytraumatic nature of these injuries (2). Furthermore, central neuropathic pain in general is often intractable to treatment; current therapies including opioids only provide ~50% pain relief in 1 out of 2-3 people (3) and the therapies are even less effective in military blast SCI due to the complexity of the injury (4). This level of treatment efficacy is unacceptable for war fighters, military personnel, veterans, and citizens as a whole. SCI patients are almost universally treated with opioids as a first-line treatment, but recent evidence in the animal literature and recently in the clinical literature indicates that opioid administration after traumatic injury can have deleterious consequences. This proposal will test a clinically relevant therapeutic, (+)-naltrexone, that we predict will improve the efficacy of opioids for controlling SCI below-level pain while decreasing the negative consequences of opioid use. We predict that the mechanism by which (+)-naltrexone exerts at least some of its effects is via toll-like receptor 4. Thus our aim is to improve the quality of life for service-members, veterans, caretakers, and the general population.

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Introduction

Spinal cord injury (SCI) is a disabling and costly condition affecting wounded military personnel (1). SCI is also one of the leading causes of central neuropathic pain, and military personnel that sustain SCI in the field by improvised explosive devices (IEDs), grenades, gunshot wounds, etc. are at an even greater risk of developing chronic pain as well as emotional symptoms due to the polytraumatic nature of these injuries (2). Furthermore, central neuropathic pain in general is often intractable to treatment; current therapies including opioids only provide ~50% pain relief in 1 out of 2-3 people (3) and the therapies are even less effective in military blast SCI due to the complexity of the injury (4). This level of treatment efficacy is unacceptable for war fighters, military personnel, veterans, and citizens as a whole. SCI patients are almost universally treated with opioids as a first-line treatment, but recent evidence in the animal literature and recently in the clinical literature indicates that opioid administration after traumatic injury can have deleterious consequences. This proposal will test a clinically relevant therapeutic, (+)-naltrexone, that we predict will improve the efficacy of opioids for controlling SCI below-level pain while decreasing the negative consequences of opioid use. We predict that the mechanism by which (+)-naltrexone exerts at least some of its effects is via toll-like receptor 4. Thus our aim is to improve the quality of life for service-members, veterans, caretakers, and the general population.

Keywords

Spinal cord injury, central neuropathic pain, rat, opioids, morphine, (+)-naltrexone, analgesia, allodynia, hyperalgesia, toll-like receptor 4

Overall Project Summary

Task 1. Obtain approval from the University of Colorado Institute Animal Care & Use Committee

(IACUC) for all animal work in the proposal. (Timeframe: 1-2 months prior to project start).

2a. Submit animal protocol covering all animal work at Boulder for IACUC meeting at least 2 months prior to anticipated start date (here, assumed to be September 1, 2013), hence July 2013 meeting or before. If revision required, submit for August 2013 meeting at latest.

Task 1 has been completed.

Milestone 1: Animal protocol is approved to allow funding to be received & to allow the project to start.

Milestone 1 has been completed.

Task 2. Purchase of, and corresponding training for operation of and maintenance of the MASCIS impactor, for contusion spinal cord injury.

2a. Purchase of MASCIS (submission of sole source letter, ordering of equipment, and awaiting delivery). Actual effort of team member will be minimal for this task, though we intend to order early to ensure delivery and training is in place such that we can proceed with Aim III when ready.

2b. Team member will attend the training offered by W. M. Keck Center for Collaborative Neuroscience. While the schedule for this training is not available as of yet, it is offered quarterly and will be taken during the quarter the impactor is received. As the impactor will be received during one of the tasks below, the course is only ~2.5 days, and will be scheduled such the departure of one team member at that time doesn't interfere with ongoing experiments.

Task 2a has been completed.

Task 2b: We have received the impactor and have begun training on caring for and maintaining the equipment. A technician in the lab is familiar with the impactor, but does not have the knowledge to train other personnel on it. We have hired a new Post-Doc, Andrew Gaudet, who is an expert in using this device as well as all aspects of the rodent spinal cord contusion model. We are excited to have Dr. Gaudet join our group given his extensive experience and expertise appropriate to this project. Andrew will not be able to begin work in our lab until January 1st, 2015 given his obligations to his present post-doctoral position at The Ohio State University.

Task 3. Aim I: Does co-administration of the TLR4 antagonist (+)-naltrexone with morphine prevent detrimental effects of morphine when this opioid is administered *shortly after* dorsal root avulsion, during the acute stages of injury?

3b. SNAP surgery and testing (Hargreaves, motor function and constipation tests during the first week post-surgery with co-administration of morphine and (+)-naltrexone (vs. vehicles) starting 1 or 24 hr post surgery; von Frey and motor function testing weekly starting on day 8; Unblinding of data & data analysis) (Timeframe: September 2013 – February 2014)
3c. Histology: tissue processing (slicing staining, image collection, image analysis, and data analysis) (Timeframe: March 2014- May 2014)

In task 3b, we have had many set-backs, some completely out of our control. The first was that our elevator for moving the animals up and down between various procedure and behavior rooms was broken and needed to be replaced, involving much drilling, destruction and re-construction. This created loud noise, strange smells (the elevator directly adjoins the animal colonies and associated behavioral testing rooms), and lots of vibration that were not conducive to behavioral testing. Thus, during this time, we were not able to run studies. Similarly, a large electrical switch panel for our building also needed to be replaced. Again, this consisted of loud noise, strange smells, and lots of vibration. This project was postponed and re-started multiple times, delaying studies for a few months as we could not have animals present during this project so delays in the project resulted in delays in re-establishing animals in the colony. Finally, due to a new faculty hire, we were required to switch behavioral testing suites. It takes time to set up new behavioral testing rooms with the correct lighting, equipment, etc, and then we had to run pilot animals (not billed to this DoD project) in the new space to be sure that our effects were comparable to those in the old space. In addition, the main person working on this project, Amanda Ellis, graduated and is moving on and will no longer be working on this project. Andrew Gaudet will be taking over, but due to circumstances beyond our control in his current lab, he will not be able to begin working with us until January 1st, 2015. There is a technician and another student that will keep the project going during this period, but the work will be much slower than anticipated as they are not fully trained on and familiar with everything that needs to be done for the project. We have also had an extremely difficult time getting a constipation model up and running. We have tried multiple methods and have consulted with others who do this type of research and have determined that these studies are confounded and cannot produce valid data. This will be discussed more in depth below with each of the presented constipation studies.

For task 3c, we have been trying to work out a fast-blue/cresyl violet staining method. We have tried combining our two different in-house protocols to no avail. We contacted Dr. Hook from the University of Texas A&M, who is an expert in this technique, and she was able to help us get a successful protocol in place.

Task 3a. Here we administered 7 days of 1x daily 10 mg/kg subcutaneous morphine/saline/(+)-naltrexone (whichever combination was appropriate as stated in the SOW) beginning 1 hr or 24 hr post-surgery and behaviorally tested the rats for mechanical allodynia using von Frey (Figures 1 and 2), analgesia using Hargreaves (Figures 3 and 4), and locomotor recovery using the ladder test (Figure 5). The groups are not complete and are currently being filled in, thus significance is not reported.

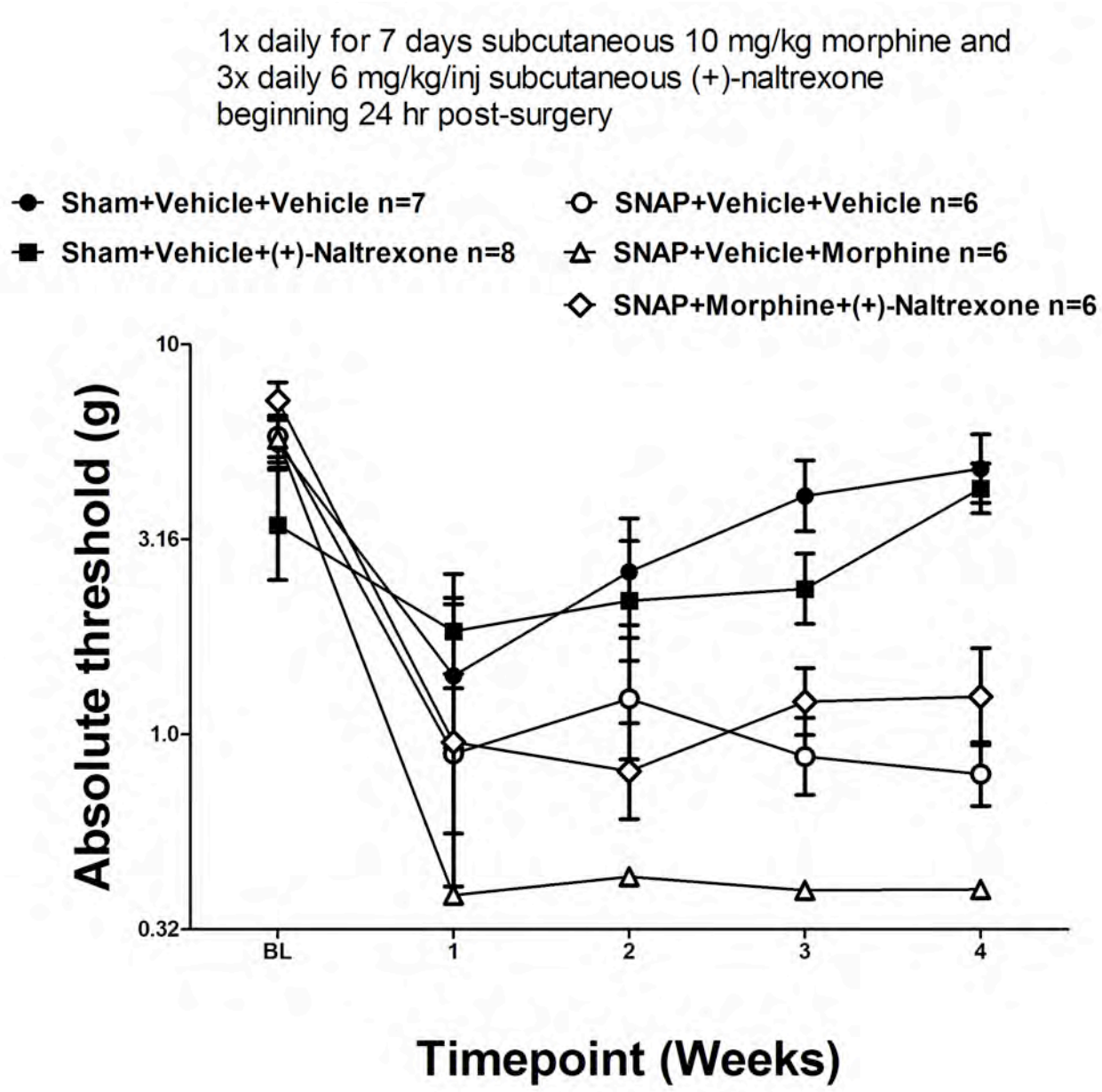

Figure 1. (+)-Naltrexone blocks morphine amplification of mechanical allodynia in SNAP rats when administered beginning 24 hrs post-surgery.

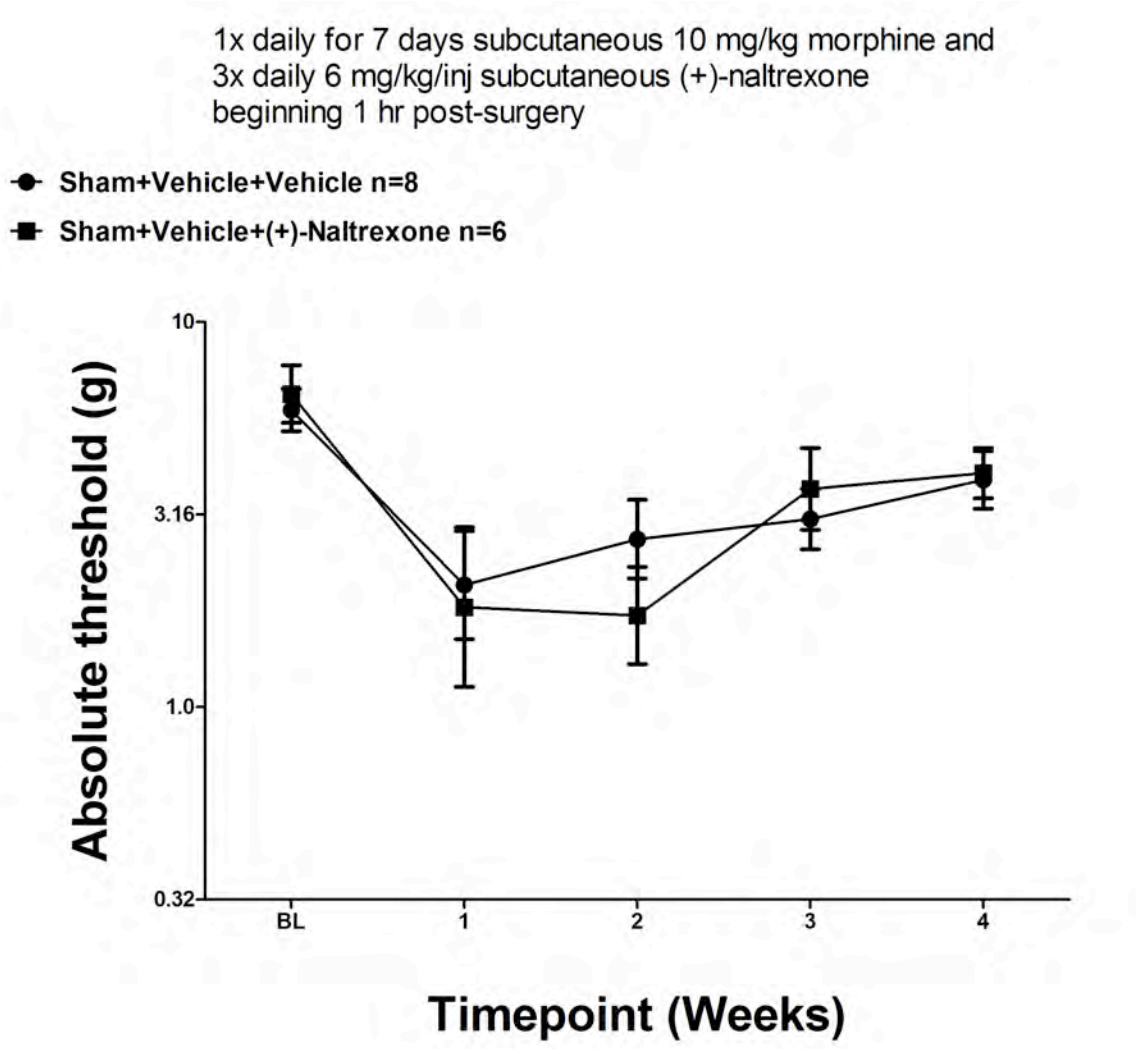

Figure 2. (+)-Naltrexone does not have any effect on mechanical allodynia in sham operated rats

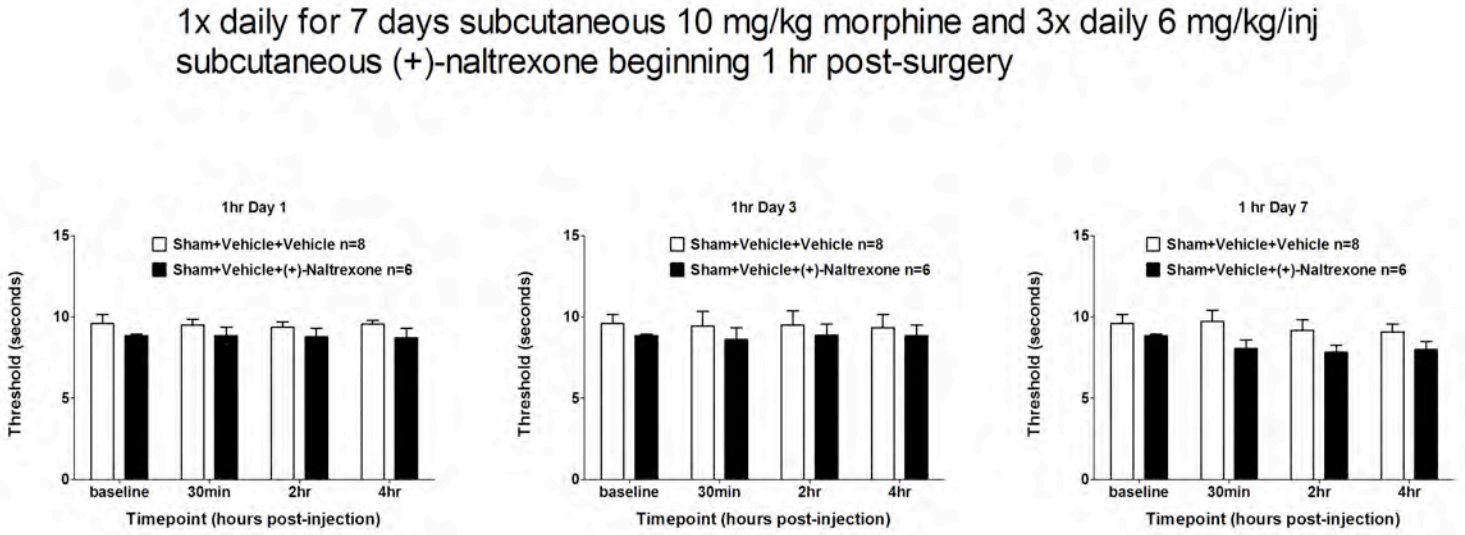

Figure 3. (+)-Naltrexone administered beginning 1 hr post-surgery does not have any effect on analgesia in sham operated rats

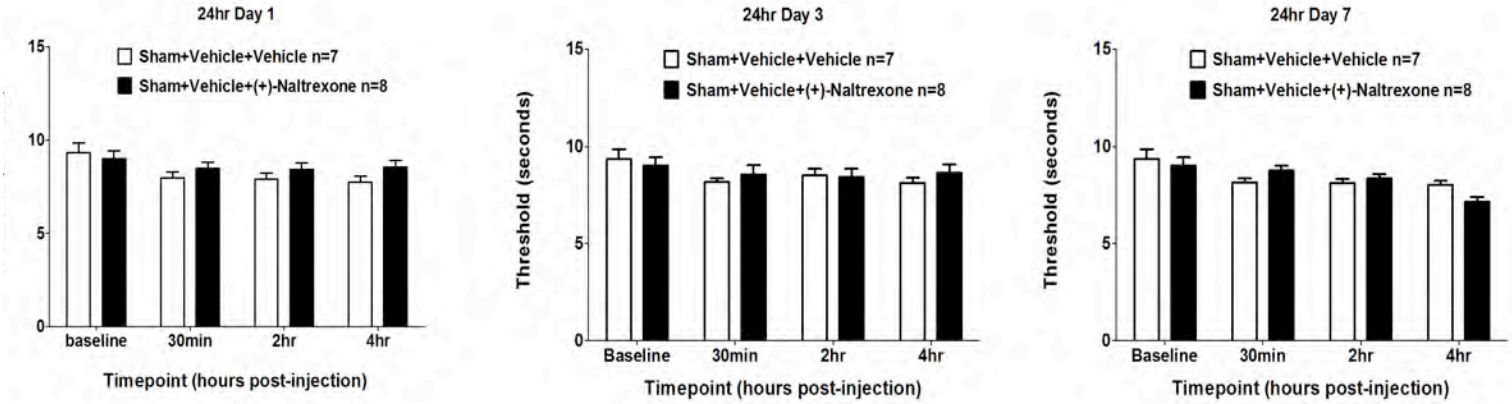

Figure 4. (+)-Naltrexone administered beginning 24 hr post-surgery does not have any effect on analgesia in sham operated rats

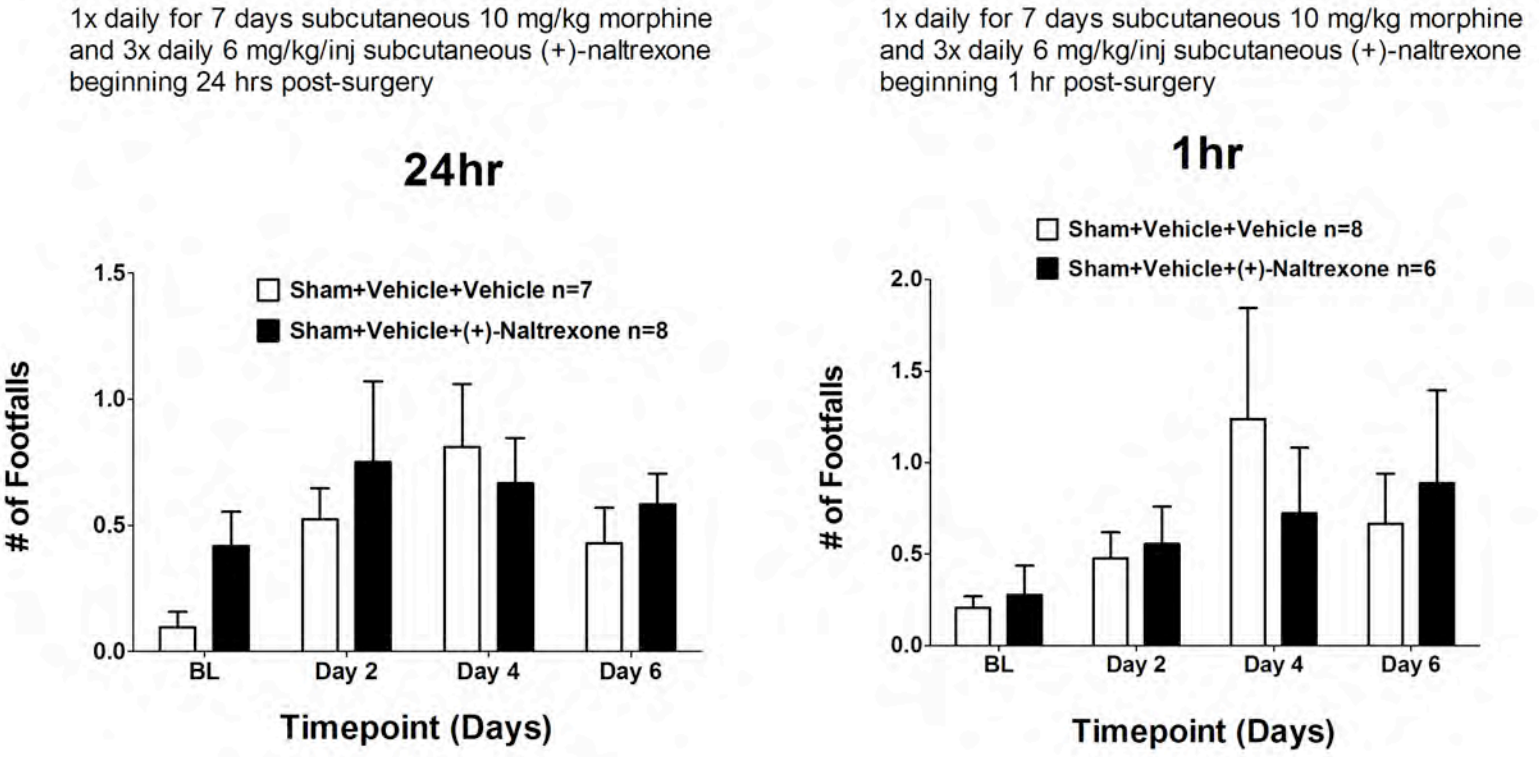

Figure 5. (+)-Naltrexone does not have any effect on locomotor behavior in sham operated rats

Task 3a. Constipation study #1

Here we just wanted to get an idea of whether or not morphine induced constipation in our SCI model. For 3 days prior to surgery, at approximately 9:30am, the rats were moved to a clean cage. At approx. 9:00am the following morning, the total amount of boli for each rat was weighed and recorded. The weights over these three days were averaged for each rat to give a baseline. Similarly, on the day after surgery, rats were moved to a clean cage and at the appropriate 24 hr timepoint, the rats were given a subcutaneous injection of 10 mg/kg morphine or equivolume saline. The next morning at approx. 9:00am, the total amount of boli for each rat was weighed and recorded. This was repeated each day for the 7 day course of morphine administration. The weights over the 7 days were averaged for each rat. Figure 6 below shows that rats that received morphine had significantly less boli weight compared to baseline.

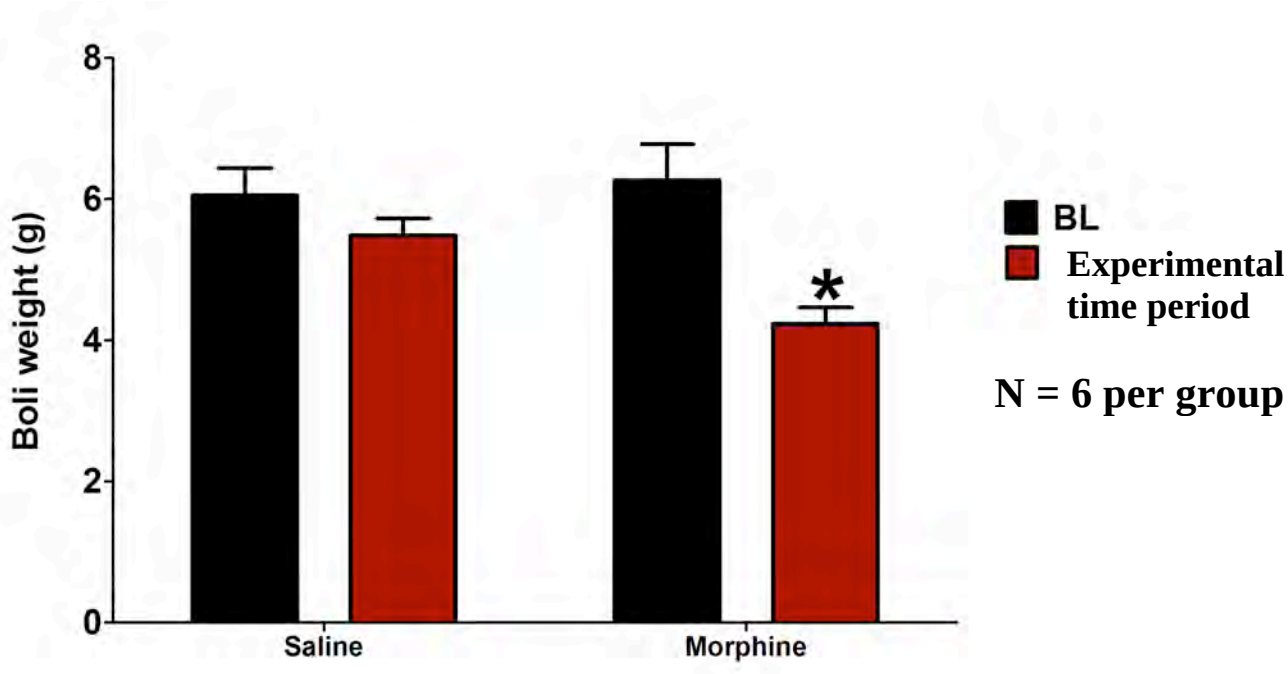

Figure 6. Morphine (10 mg/kg s.c. for 7 days) significantly decreased boli weight compared to baseline ($p = 0.0227$) in spinal cord injured rats (n=6). There were no differences between baseline and the experimental time period in spinal cord injured rats that received saline vehicle ($p > 0.05$).

Task 3a. Constipation study #2

We next started with a blue dyed highly palatable rat chow. The first batch of chow was not dyed dark enough to turn the feces blue, and the company made us a new batch for no cost, which finally did turn the feces blue. In order to see whether or not the blue chow method was reliable, we only tested a group of n = 6 SCI rats that received saline vehicle. Here we administered the blue chow 30min prior to the saline injection at 9:00am, but what we found is that each rat ate the chow at a very different rate, and also ate variable amounts. Each rat was given 30 grams of total blue chow and all rats received s.c. 1 ml/mg saline. This protocol resulted in very variable time to first blue boli, as shown below in Table 1. We

repeated this experiment two more times with similar results, and determined that this was not an ideal method to measure constipation in these rats. We proposed in the original grant proposal that if the blue palatable chow did not work, we would gavage Evan's blue dye instead, which is detailed below in Constipation Study #3.

Day 1

Rat #	Time Given Chow	Time all chow eaten	Time of first blue boli
1	8:30	9:00	17:00
2	8:30	10:00	None
3	8:30	10:00	17:20
4	8:30	11:00	None
5	8:30	9:00	None
6	8:30	11:00	None

Day 2

Rat #	Time Given Chow	Time all chow eaten	Time of first blue boli
1	8:00	9:00	13:00
2	8:00	9:00	13:00
3	8:00	10:00	14:00
4	8:00	10:00	13:00
5	8:00	11:00	None
6	8:00	9:00	10:00

Day 3

Rat #	Time Given Chow	Time all chow eaten	Time of first blue boli
1	8:00	9:00	13:00
2	8:00	10:00	12:00
3	8:00	9:00	11:00
4	8:00	10:00	14:00
5	8:00	11:00	14:00
6	8:00	10:00	15:00

Day 4

Rat #	Time Given Chow	Time all chow eaten	Time of first blue boli
1	8:00	9:00	12:00
2	8:00	10:00	12:00
3	8:00	10:00	12:00
4	8:00	9:00	11:00
5	8:00	1:00	12:00
6	8:00	11:00	12:00

Day 5

Rat #	Time Given Chow	Time all chow eaten	Time of first blue boli
1	7:45	11:00	18:00
2	7:45	9:00	None
3	7:45	9:00	18:00
4	7:45	9:00	17:00
5	7:45	9:00	None
6	7:45	10:00	None

Day 6

Rat #	Time Given Chow	Time all chow eaten	Time of first blue boli
1	8:00	9:00	17:00
2	8:00	9:00	None
3	8:00	10:00	None
4	8:00	10:00	None
5	8:00	10:00	17:00
6	8:00	10:00	18:00

Day 7

Rat #	Time Given Chow	Time all chow eaten	Time of first blue boli
1	8:00	10:00	None
2	8:00	9:00	13:00
3	8:00	11:00	None
4	8:00	10:00	None
5	8:00	9:00	17:00
6	8:00	11:00	14:00

Table 1: Large variability in blue rat chow boli data

Task 3a. Constipation Study #3

Since SCI rats have a small part of their vertebrae missing, their backs are very fragile and you cannot gavage them in the usual manner. Instead of holding them by their shoulders and letting their hind legs dangle, their hind quarters must be supported. The only way to do this without hurting the rat, given that their lumbar spinal cord is no longer protected by overlying bone, is to lay the rat flat on its stomach. In order to be able to do this, the rats must be put under very light isofluorane anesthesia (approx. 2 min) in a padded foam bell jar. Although we mastered this method, the results as shown below in Table 2 were still variable and inconsistent (all animals received saline and then either saline or (+)-naltrexone). In addition, it was very hard on the animals to go under isoflurane and get a gavage 3x/wk on top of all of the other behavioral testing and injections. The health of the animals was a big concern, and these rats didn't even receive morphine, only saline. We anticipate even more concerning health issues were we to add morphine. The gavages also negatively affected the other behavioral tests. The rats were quite stress, anxious, and took longer than normal (i.e. a non gavaged rat) to calm down for both Hargreave's and von Frey testing. They were also very jumpy and hyper-responsive on these behavioral tests. The rats were very reluctant to perform the horizontal ladder task, which we usually do not have issues with. The rats were also more vocal than normal on all of the behavioral tests. We concluded that the constipation studies should be dropped, because after spending much time and money working out these methods, the constipation studies actually severely compromise the health of the animals and confound the rest of the data (especially since all of the behavioral tests and constipation testing are done in the same animals).

Day 2

Rat #	Time of Surgery	Time of Gavage	Time of first blue boli (pm)	Total Time (hrs)	Sal/(+)-Nal	Mean Saline
1	10:17am	9:00am	3:00	6	s	5.5625
2	10:43am	9:00am	1:45	4.75	S	Mean +Naltrexone
3	11:10am	9:00am	1:45	4.75	N	5.75
4	11:35am	9:00am	2:00	5	S	STD Saline
5	1:11pm	12pm	6:00	6	N	0.826009483
6	1:37pm	12pm	Died from iso		N	STD +Nal

7	2:02pm	12pm	6:30	6.5	S	0.901387819
8	2:25pm	12pm	6:30	6.5	N	

Day 2/4

Rat #	Time of Surgery	Time of Gavage	Time of first blue boli (pm)	Total Time (hrs)	Sal/(+)-Nal	Mean Saline
1	10:17am	8:30am	2:45	6.25	S	5.709090909
2	10:43am	8:30am	2:30	6	S	Mean +Naltrexone
3	11:10am	8:30am	2:00	5.5	N	5.168181818
4	11:35am	8:30am	2:00	5.5	S	STD Saline
5	1:11pm	12:45pm	6:30	5.75	N	1.302270744
7	2:02pm	12:45pm	6:00	5.25	S	STD +Nal
8	2:25pm	12:45pm	6:00	5.25	N	0.940019342
9	10:45am	8:40am	2:15	5.5	N	
10	11:15am	8:40am	2:15	5.5	S	
11	11:41am	8:40am	2:30	5.75	S	
13	1:00pm	12:55pm	5:00	4.9	N	
14	1:37pm	12:55pm	4:00	3.9	N	
15	2:12pm	12:55pm	6:30	5.5	N	
16	2:42pm	12:55pm	5:30	4.5	S	
17	10:52am	8:50am	3:00	6.8	N	
18	11:27am	8:50am	3:00	6.8	S	
19	12:00pm	8:50am	2:30	5.5	N	
20	12:29pm	8:50am	5:30	8.75	S	
21	1:41pm	1:05pm	4:15	3.25	N	
22	2:05pm	1:05pm	5:30	4.5	S	
23	2:40pm	1:05pm	6:00	5	N	
24	3:05pm	1:05pm	5:00	4	S	

Day 2/4/6

Rat #	Time of Surgery	Time of Gavage	Time of first blue boli (pm)	Total Time (hrs)	Sal/(+)-Nal	Mean Saline
1	10:17am	8:30am	2:30	6	S	5.571428571
2	10:43am	8:30am	1:45	5.25	S	Mean +Naltrexone
3	11:10am	8:30am	1:45	5.25	N	5.333333333
4	11:35am	8:30am	2:30	6	S	STD Saline
5	1:11pm	12:00pm	6:30	6.5	N	1.051642706
7	2:02pm	12:00pm	6:00	6	S	STD +Nal
8	2:25pm	12:00pm	6:30	6.5	N	1.046536237
9	10:45am	8:40am	2:30	6.25	N	
10	11:15am	8:40am	12:30	3.75	S	
11	11:41am	8:40am	2:00	5.25	S	
13	1:00pm	12:10pm	5:15	5	N	
14	1:37pm	12:10pm	4:15	4	N	

15	2:12pm	12:10pm	6:30	6.25	N
16	2:42pm	12:10pm	5:15	5	S
17	10:52am	8:50am	3:00	6.25	N
18	11:27am	8:50am	2:30	6.5	S
19	12:00pm	8:50am	2:00	5.25	N
20	12:29pm	8:50am	5:15	8.5	S
21	1:41pm	12:20pm	4:00	3.75	N
22	2:05pm	12:20pm	5:15	5	S
23	2:40pm	12:20pm	6:00	6.25	N
24	3:05pm	12:20pm	5:15	5	S
25	10:37am	9:00am	2:00	5	S
26	11:08am	9:00am	1:02	4	N
27	11:33am	9:00am	2:30	5.5	S
28	11:55am	9:00am	2:00	5	N
29	1:21pm	12:30pm	6:00	5.5	S
30	1:47pm	12:30pm	6:30	6	N
31	2:13pm	12:30pm	5:15	4.75	S
32	2:40pm	12:30pm	4:20	3.75	N

Day 4/6

Rat #	Time of Surgery	Time of Gavage	Time of first blue boli (pm)	Total Time (hrs)	Sal/(+)-Nal	Mean Saline
9	10:45am	8:30am	2:00	5.5	N	5.545454545
10	11:15am	8:30am	12:30	4	S	Mean +Naltrexone
11	11:41am	8:30am	2:30	6	S	4.884615385
13	1:00pm	12:00pm	5:00	5	N	STD Saline
14	1:37pm	12:00pm	4:00	4	N	1.844319699
15	2:12pm	12:00pm	6:15	6.25	N	STD +Nal
16	2:42pm	12:00pm	5:15	5.25	S	0.884419001
17	10:52am	8:40am	3:15	6.5	N	
18	11:27am	8:40am	2:45	6	S	
19	12:00pm	8:40am	2:15	5.5	N	
20	12:29pm	8:40am	5:00	7.75	S	
21	1:41pm	12:10pm	4:30	4.25	N	
22	2:05pm	12:10pm	5:00	4.75	S	
23	2:40pm	12:10pm	6:30	6.25	N	
24	3:05pm	12:10pm	5:15	5	S	
25	10:37am	8:50am	2:15	5.5	S	
26	11:08am	8:50am	1:30	4.75	N	
27	11:33am	8:50am	2:00	5.75	S	
28	11:55am	8:50am	2:00	5.75	N	
29	1:21pm	12:20pm	6:15	6	S	
30	1:47pm	12:20pm	6:00	5.75	N	
31	2:13pm	12:20pm	5:30	5	S	
32	2:40pm	12:20pm	4:30	4	N	

Day 6						
Rat #	Time of Surgery	Time of Gavage	Time of first blue boli (pm)	Total Time (hrs)	Sal/(+)-Nal	Mean Saline
25	10:37am	8:00am	2:00	6	S	6
26	11:08am	8:02am	1:00	5	N	Mean +Naltrexone
27	11:33am	8:05am	2:00	6	S	5.75
28	11:55am	8:07am	3:00	7	N	STD Saline
29	1:21pm	12:10pm	7:00	7	S	0.816496581
30	1:47pm	12:12pm	6:00	6	N	STD +Nal
31	2:13pm	12:14pm	5:00	5	S	0.957427108
32	2:40pm	12:16pm	5:00	5	N	

Table 2: Constipation studies with gavage

Task 3b. Histology studies: Here we took ~ 5mm of spinal cord surrounding the injury site and sliced at 20 um and then stained with luxol fast blue and cresyl violet to determine the extent of damage. In order to work out the staining method, we used spinal cords from sham operated animals first. As mentioned above, we had problems getting the method to work when we simply fused our two individual protocols for luxol fast blue and cresyl violet together. We sought the advice of an expert in this type of staining and analysis, and based on her recommendations we were finally able to get a successful staining protocol in place. We are currently working on the analyses. Figure 7A depicts the left dorsal horn of a spinal cord section from a saline treated sham animal at the center of the injury site and Figure 7B depicts the left dorsal horn of a spinal cord section from a (+)-naltrexone treated sham animal at the center of the injury site.

A B

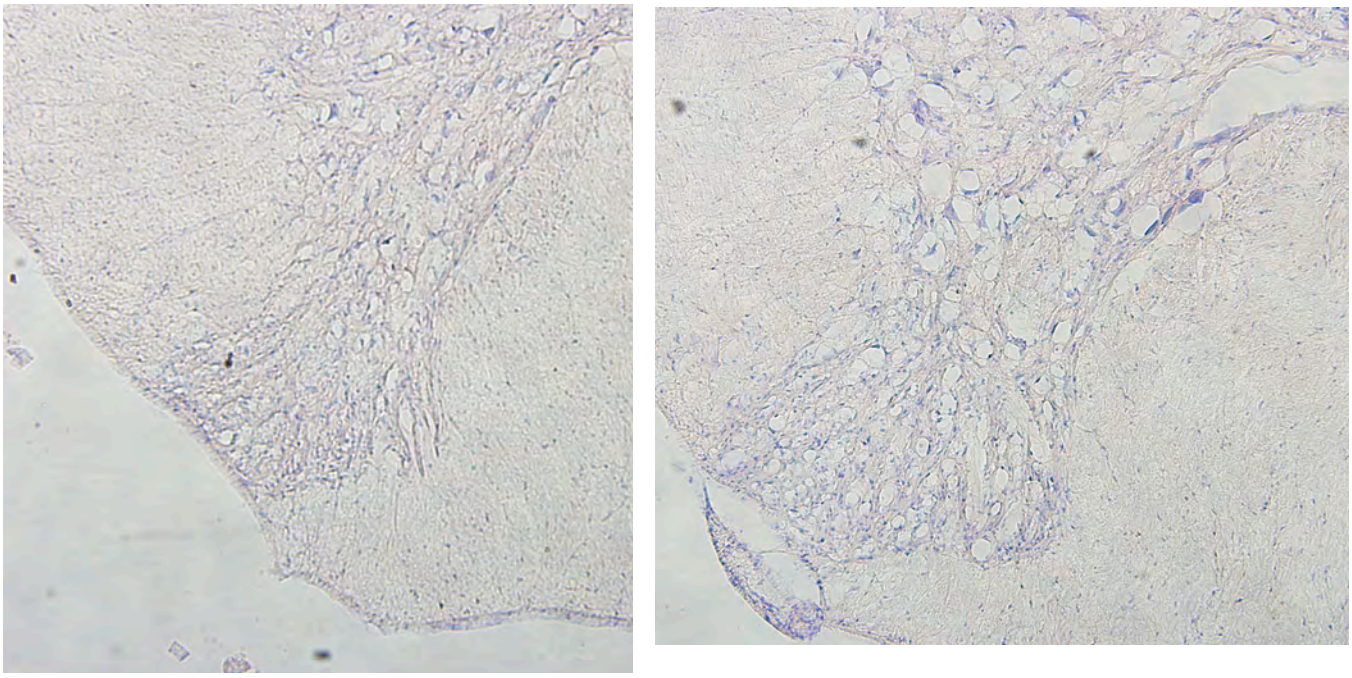

Figure 7. Successful staining using a fast blue and cresyl violet staining method in the dorsal spinal cord of sham rats that received saline (A) and (+)-naltrexone (B)

<u>Task 5.</u> Aim II: Does co-administration of the TLR4 antagonist (+)-naltrexone with morphine prevent detrimental effects of morphine when morphine is administered *<u>weeks after (6 weeks)</u>* dorsal root avulsion, after *development of below-level neuropathic pain*?

5b. SNAP surgery and testing (baseline all measures before initiation of week-long drug treatment; Hargreaves, motor function and constipation tests during the week of co-administration of morphine and (+)-naltrexone starting 6 weeks post surgery; von Frey and motor testing weekly starting again after completion of course of drug treatment; Unblinding of data & data analysis) (Timeframe: May 2014 – November 2014)

5c. Histology: tissue processing (slicing staining, image collection, image analysis, and data analysis) (Timeframe: November 2014- December 2014)

We have ordered animals to begin task 5.

Key Research Accomplishments

- (+)-Naltrexone blocks morphine-induced amplification of spinal neuropathic avulsion pain when co-administered with morphine beginning 24 hrs post-surgery
- (+)-Naltrexone does not have any behavioral effect on sham operated control animals

CONCLUSION

We continue to make good progress and have maintained, as close as possible given problems encountered during this period, the required outputs and data collection according to the statement of work. (+)-Naltrexone continues to present as a novel compound, blocking the deleterious effects of administering morphine shortly after SCI. This is important clinically because virtually all traumatic injury patients, and many neuropathic pain patients, with both central and peripheral neuropathies, receive opioids at some point. We also know that (+)-naltrexone on its own does not affect behavior in sham operated rats, which is important because it suggests that you need pain/inflammation present in order to observe the positive effects and (+)-naltrexone itself is not causing any negative effects. Taken together, all of these data suggest that (+)-naltrexone would be a successful new neuropathic pain treatment and could be administered along with opioids to create a better treatment than either drug on its own. At the same time, it is important to continue investigating the underlying mechanisms of this remarkable therapeutic compound in order to use it most effectively. We thank the Department of Defense for their continued support of the project and hope they find the outcome of the project to date exciting and novel with potential clinical relevance down the road.

PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a. Nothing to report as of yet
b. Invited talk at Keystone Pain Conference in Keystone, CO June 13-20th 2014 Title: Morphine Following Spinal Cord Injury Amplifies Pain and Neuroinflammation

INVENTIONS, PATENTS AND LICENSES

Nothing to report as of yet

REPORTABLE OUTCOMES

Nothing to report as of yet

OTHER ACHIEVEMENTS

The graduate student working on this project, Amanda Ellis, received her PhD on June 6th, 2014.
We have hired a Professional Research Assistant with excellent surgical skills and experience with the spinal cord contusion model who is training on the avulsion surgery and procedures.
We have hired a Research Associate from The Ohio State University currently in Dr. Philip Popovich's laboratory, with extensive spinal cord contusion experience.

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APPENDICES

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